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Different protocols of physical exercise produce different effects on synaptic and structural proteins in motor areas of the rat brain

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ABSTRACT

The plastic brain responses generated by the training with acrobatic exercise (AE) and with treadmill exercise (TE) may be different. We evaluated the protein expression of synapsin I (SYS), synaptophysin (SYP), microtubule-associated protein 2 (MAP2) and neurofilaments (NF) by immunohistochemistry and Western blotting in the motor cortex, striatum and cerebellum of rats subjected to TE and AE. Young adult male Wistar rats were divided into 3 groups: sedentary (Sed) (n=15), TE (n=20) and AE (n=20). The rats were trained 3 days/week for 4 weeks on a treadmill at 0.6 km/h, 40 min/day (TE), or moved through a circuit of obstacles 5 times/day (AE). The rats from the TE group exhibited a significant increase of SYS and SYP in the motor cortex, of NF68, SYS and SYP in the striatum, and of MAP2, NF and SYS in the cerebellum, whereas NF was decreased in the motor cortex and the molecular layer of the cerebellar cortex. On the other hand, the rats from the AE group showed a significant increase of MAP2 and SYP in the motor cortex, of all four proteins in the striatum, and of SYS in the cerebellum. In conclusion, AE induced changes in the expression of synaptic and structural proteins mainly in the motor cortex and striatum, which may underlie part of the learning of complex motor tasks. TE, on the other hand, promoted more robust changes of structural proteins in all three regions, especially in the cerebellum, which is involved in learned and automatic tasks.

1. Introduction

Exercise induces several positive effects in the central nervous system of humans (Dustman et al., 1990; Lupinacci et al., 1993) and animals (Ferreira et al., 2010, 2011; Kleim et al., 1996, 1997; Real et al., 2010), such as improved learning, memory and plasticity (Lambert et al., 2005; van Praag et al., 1999a; Vaynman et al., 2006), increased neuronal activation (Holschneider et al., 2007; Lewis et al., 2007) and enhanced neurogenesis (Ferreira et al., 2011; van Praag et al., 1999a; van Praag et al., 1999b). Changes of neurotransmitters and their receptors (Del Arco et al., 2007b; Real et al., 2010) and of the expression of genes related to synaptic plasticity (Ferreira et al., 2010; Molteni et al., 2002), which are responsible for changing the number, structure and

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function of neurons (Ang et al., 2006; van Praag et al., 1999a), have also been observed.

Voluntary, treadmill and acrobatic exercise (VE, TE and AE, respectively) induce distinct plastic responses in different brain regions (Kleim et al., 2002; Klintsova et al., 2004; Vaynman et al., 2006). Studies using electron microscopy have shown that animals submitted to all three of these modalities presented plastic changes in the cerebellum and motor cortex. In the cerebellum, for example, there was an increase of the number of synapses of parallel fibers and Purkinje cells and an increased number of synapses per Purkinje cell, higher in the AE than the VE. This increase persisted with time (Black et al., 1990; Kleim et al., 1997, 1998b). Furthermore, it was observed that the AE group completed the designated tasks in less time, indicating a substantial gain of motor abilities (Kleim et al., 1996, 2002).

A series of studies have focused on the participation of synaptic (Ferreira et al., 2010; Lambert et al., 2005; Vaynman et al., 2004) and structural proteins (Derksen et al., 2007; Ferreira et al., 2010, 2011) in exercise-induced plastic changes. Microtubule-associated protein 2 (MAP2) and synaptophysin (SYP) have been studied after short-term AE and increases of SYP have been observed in the motor cortex, possibly related to the first 5 days of learning a motor skill. MAP2 changes, on the other hand, did not seem to increase according to the duration or difficulty of the acrobatic training (Derksen et al., 2007). Other authors have also reported increases of NF68, SYS and SYP in the striatum after short-term TE, which was accompanied by increases of SYS and NF68 in the cerebellum and a decrease, followed by increase, of NFs in the motor cortex (Ferreira et al., 2010). AE also induces increase of neurotrophins, such as BDNF, in the motor cortex and cerebellum (Klintsova et al., 2004), whereas TE increases BDNF in the striatum (Tajiri et al., 2010) and cortex (Rasmussem et al., 2009). A close relation between BDNF and the synaptic vesicle proteins SYS and SYP was observed by Vaynman et al. (2006), which demonstrated that the blockade of BDNF actions inhibited the VE-induced increase of these synaptic proteins. Learning a new motor skill (AE) can induce dendritic reorganization, synaptogenesis and changes of synaptic morphology, all of which require protein synthesis (Derksen et al., 2007). Training rats on a novel motor task can be an effective option to study the brain motor circuits in response to learning. A distinction has been made between tasks that require a high amount of attentional guidance (skilled training, internally guided) and those that do not (overlearned, automatic, and externally guided), and each has been linked with different patterns of functional brain activation and recruitment of different motor circuits (Adkins et al., 2006; Holschneider et al., 2007). In addition, it is becoming increasingly recognized that no single exercise paradigm is likely to fulfill all therapeutic needs (Cotman and Berchtold, 2007).

Various exercise protocols have been used to study its beneficial effects, such as acrobatic exercise, wheel running, and treadmill exercise of low to high intensity, continuous or intermittent, and of short and long duration (Ferreira et al., 2010; Kleim et al., 1997; Real et al., 2010). However, depending on the intensity of training, exercise could be, instead of beneficial, deleterious to the brain, by causing increased free radicals, cytokine production and excitotoxicity (Arida et al., 2011). Enriched environments have also been used, and all these modalities have the purpose of investigating specific exercise-induced plastic changes that occur in different brain regions (Del Arco et al., 2007a; Mora et al., 2007). We evaluated here if TE and AE could change structural and synaptic proteins in the same way in different motor areas of the rat brain. This could be used to help understand how different regions of the brain contribute to the motor learning process triggered by

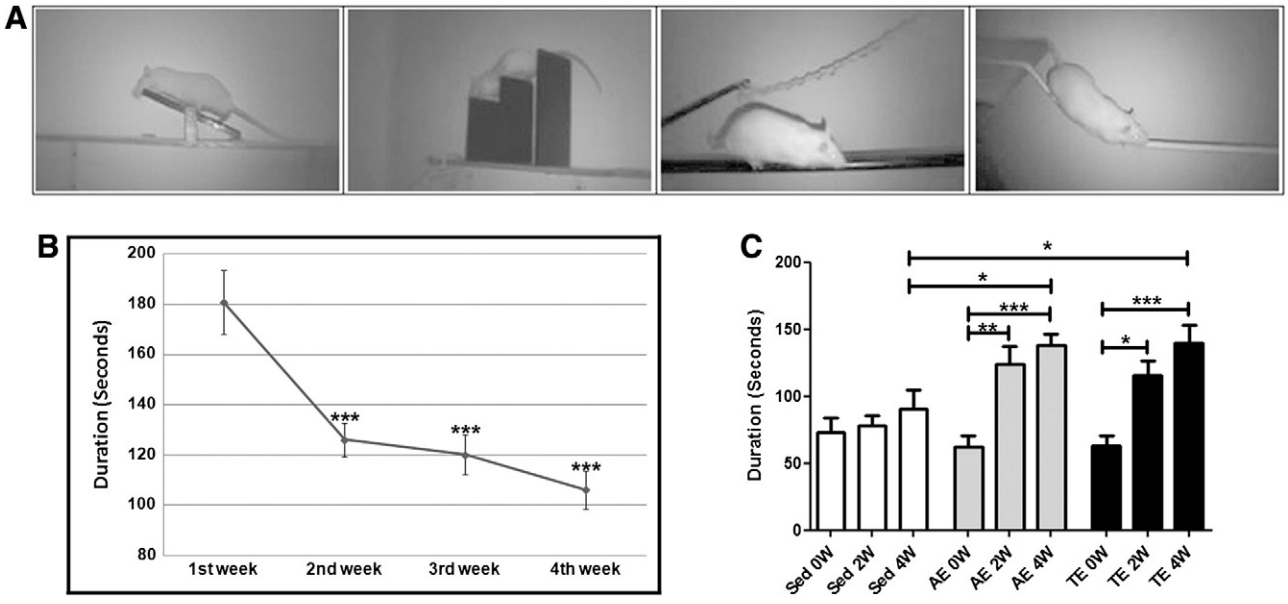

Fig. 1 – Behavioral evaluation. (A) See-saw, balance beams rope ladder and thin dowels as examples of the obstacles of the acrobatic circuit. (B) Evaluation of the acrobatic performance considering the recorded time to complete the acrobatic circuit each week. (C) Balance and coordination evaluation using the Rotarod considering the time spent on the equipment on the first day (0 W), on the second week (2 W) and on the fourth week (4 W) of the protocols.

different exercise modalities and how this could contribute to the development of therapeutic strategies.

2. Results

2.1. Balance and coordination

Using the Rotarod to evaluate motor skills, we observed that all groups presented the same level of motor skills in the initial evaluation. On the second week of training, we could observe that both exercise groups significantly increased the time spent on the Rotarod (AE: $p<0.01$; TE: $p<0.001$) and this increase persisted until the fourth week of training (AE: $p<0.05$; TE: $p<0.001$) (Fig. 1). We did not observe, however, differences between the two exercise modalities used here in this evaluation.

2.2. Acrobatic performance

After 4 weeks of AE, the animals presented a significant reduction of the time necessary to complete the AE task ($p<0.01$) (Fig. 1). This reduction was observed after the second week of AE training and persisted for all evaluation periods until the end of the 4 weeks of training. We also observed that the animal hesitated less from one obstacle to the other and consequently less manual guidance was used.

2.3. Protein expression

Protein expression of MAP2, NF, SYS and SYP in the three brain regions studied here was evaluated using immunohistochemistry and Western blotting. The results of these analyses are summarized in Table 1 and described in detail below.

Table 1 – Effects of different modalities of exercise upon structural and synaptic protein expression in motor regions of the rat brain.

Brain region	Protein	Western blotting			Protein	Immunostaining					
		Sed	AE	TE		Sed		AE		TE	
		Mean (±SEM)	Mean (±SEM)	Mean (±SEM)		Mean (±SEM)		Mean (±SEM)		Mean (±SEM)	
Motor cortex	MAP2	1 (±0.03)	1.35 (±0.14)**	0.91 (±0.02)	MAP2	1 (±0.04)		1.29 (±0.07)**		1.07 (±0.03)	
	NF68	1 (±0.06)	0.89 (±0.15)	0.68 (±0.05)	NF	1 (±0.04)		1.08 (±0.01)		0.93 (±0.03)	
	NF160	1 (±0.05)	0.86 (±0.04)	0.44 (±0.04)***							
	NF200	1 (±0.09)	1.01 (±0.10)	0.49 (±0.06)**							
	SYS	1 (±0.04)	0.94 (±0.44)	1.13 (±0.08)	SYS	1 (±0.05)		1.02 (±0.06)		1.47 (±0.07)**	
	SYP	1 (±0.03)	0.91 (±0.03)	0.97 (±0.08)	SYP	1 (±0.03)		1.35 (±0.08)**		1.43 (±0.02)**	
Cerebellum						GL	ML	GL	ML	GL	ML
	MAP2	1 (±0.02)	1.13 (±0.14)	2.84 (±0.40)***	MAP2	1 (±0.06)	1 (±0.02)	0.91 (±0.06)	1.14 (±0.05)	1.02 (±0.03)	1.36 (±0.04)**
	NF68	1 (±0.07)	1.27 (±0.14)	1.22 (±0.07)	NF	1 (±0.09)	1 (±0.06)	1.32 (±0.07)	0.91 (±0.03)	1.43 (±0.07)*	0.84 (±0.01)*
	NF160	1 (±0.11)	0.95 (±0.07)	1.10 (±0.17)							
	SYS	1 (±0.04)	0.95 (±0.08)	1.02 (±0.08)	SYS	1 (±0.04)	1 (±0.02)	1.04 (±0.01)	1.18 (±0.03)**	1.20 (±0.03)**	1.14 (±0.01)**
	SYP	1 (±0.07)	0.97 (±0.08)	1.04 (±0.09)	SYP	1 (±0.05)	1 (±0.03)	1.13 (±0.05)	1 (±0.02)	1.13 (±0.01)	1.06 (±0.01)
Striatum						Med	Lat	Med	Lat	Med	Lat
	MAP2	1 (±0.07)	1.36 (±0.07)**	1.04 (±0.08)	MAP2	1 (±0.03)	1 (±0.18)	2.53 (±0.31)**	1.55 (±0.19)	1.63 (±0.07)	0.83 (±0.09)
	NF68	1 (±0.11)	1.44 (±0.08)*	1.86 (±0.15)***	NF	1 (±0.03)	1 (±0.04)	1.07 (±0.03)	0.88 (±0.02)	1.01 (±0.04)	1 (±0.07)
	SYS	1 (±0.07)	0.77 (±0.11)	0.93 (±0.05)	SYS	1 (±0.03)	1 (±0.06)	1.26 (±0.02)**	1.06 (±0.05)	1.31 (±0.06)**	1.12 (±0.03)
	SYP	1 (±0.07)	1.27 (±0.05)*	1.17 (±0.09)	SYP	1 (±0.04)	1 (±0.01)	1.18 (±0.05)*	1.06 (±0.01)	1.03 (±0.03)	1.14 (±0.02)**

Sed: sedentary; AE: acrobatic exercise; TE: treadmill exercise; MAP2: microtubule-associated protein 2; NF: neurofilaments; NF68: 68 kDa neurofilament; NF160: 160 kDa neurofilament; NF200: 200 kDa neurofilament; SYS: synapsin I; SYP: synaptophysin; GL: granular layer of the cerebellar cortex; ML: molecular layer of the cerebellar cortex; Med: dorsomedial striatum; Lat: dorsolateral striatum (*$p<0.05$; **$p<0.03$; ***$p<0.001$ vs. Sed group).

2.3.1. *Motor cortex*

In the motor cortex, the staining for MAP2 was present along the neuropil throughout layer V of the primary and secondary motor cortices (M1 and M2), with a significantly higher intensity of staining in the AE group ($p<0.03$). The staining of NFs, on the other hand, was observed along the neuropil and perikarya and was significantly less intense in the TE group in relation to the AE group ($p<0.05$). Protein analysis of the motor cortex corroborated with the immunohistochemistry findings, as we observed increased levels of MAP2 after AE ($p<0.03$) and decreased levels of NF160 and NF200 after TE ($p<0.001$ and $p<0.03$, respectively) with no changes for NF68 (Fig. 2).

The staining for SYS and SYP exhibited a cytoplasmic pattern throughout the motor cortex and we observed higher intensity of staining of SYS in the TE group ($p<0.03$), whereas a higher intensity of staining of SYP was observed in both exercised groups ($p<0.03$). Protein analysis, however, did not show any changes of synaptic proteins in the motor cortex (Fig. 3).

2.3.2. *Striatum*

In the striatum, we observed staining for MAP2 along the neuropil throughout the rostro-caudal striatum, especially in the medial region. The intensity of the staining for MAP2 was higher in the dorsomedial striatum of the AE group ($p<0.03$) and also in the dorsolateral striatum in relation to the TE group ($p<0.05$). As for the staining of NFs, we observed a punctate pattern gathered in bundles where axons pass throughout the region, with no differences among the groups. Protein analysis of the striatum revealed increases of MAP2 and NF68 protein levels after AE ($p<0.03$ and $p<0.05$, respectively), whereas TE increased only NF68 protein levels ($p<0.001$) (Fig. 4).

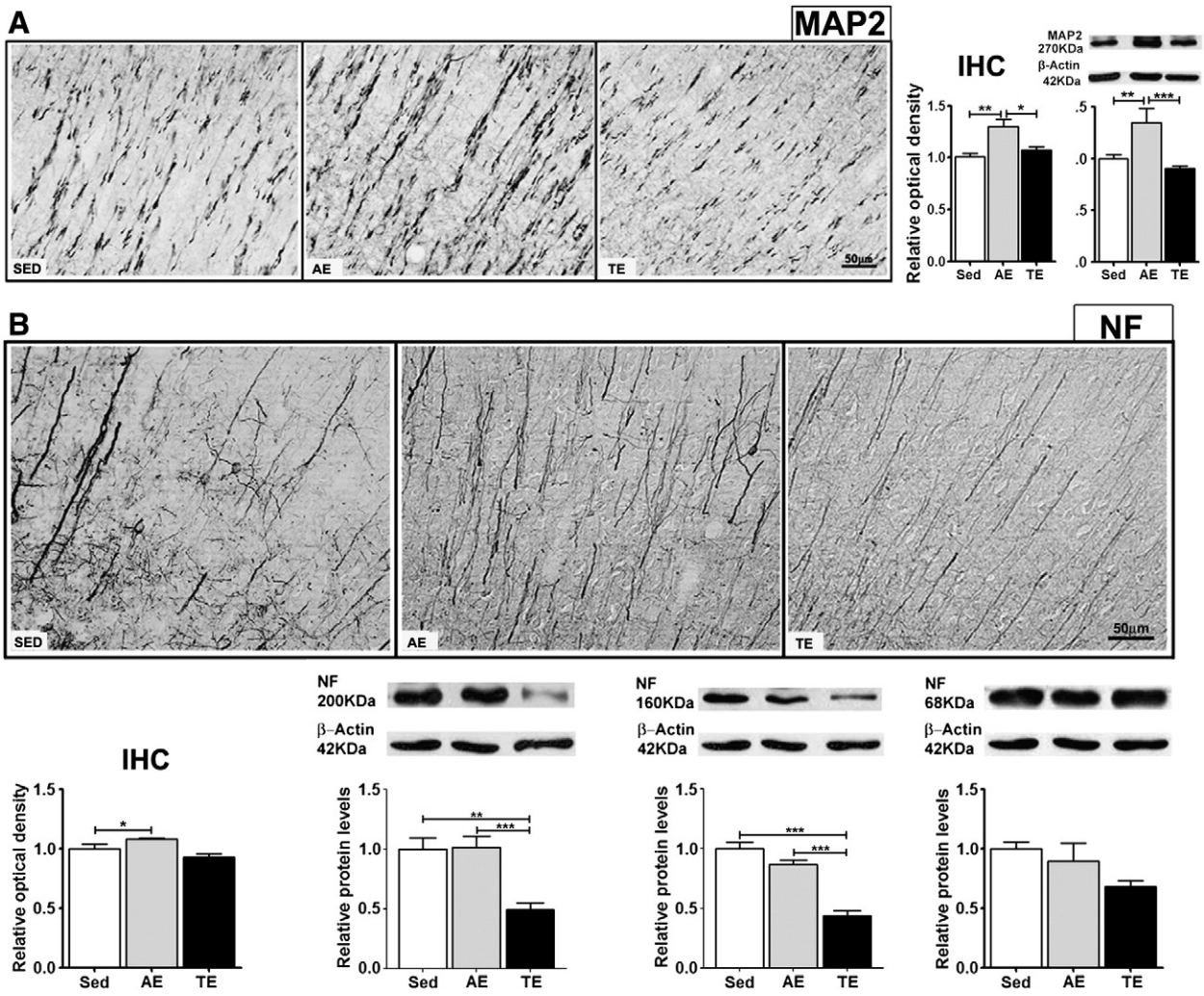

Fig. 2 – Effects of AE and TE on MAP2 (A) and NFs (B) in the rat motor cortex. Digital images of coronal sections of the motor cortex stained for MAP2 and NFs. Mean ratio of MAP2 and NFs integrated density data in the motor cortex (M1 and M2) in relation to the background optical density (N = 6–8 per group). Mean ratio of MAP2/β-actin and NF68/β-actin, NF160/β-actin, NF200/β-actin (N = 7–14 per group). Density data relative to the sedentary group (normalized) and typical immunoblots in each condition. AE: acrobatic exercise; TE: treadmill exercise; Sed: sedentary; MAP2: microtubule-associated protein 2; NF: neurofilaments; NF68: 68 kDa neurofilament; NF160: 160 kDa neurofilament; NF200: 200 kDa neurofilament; IHC: immunohistochemistry (*$p<0.05$; **$p<0.03$; ***$p<0.001$).

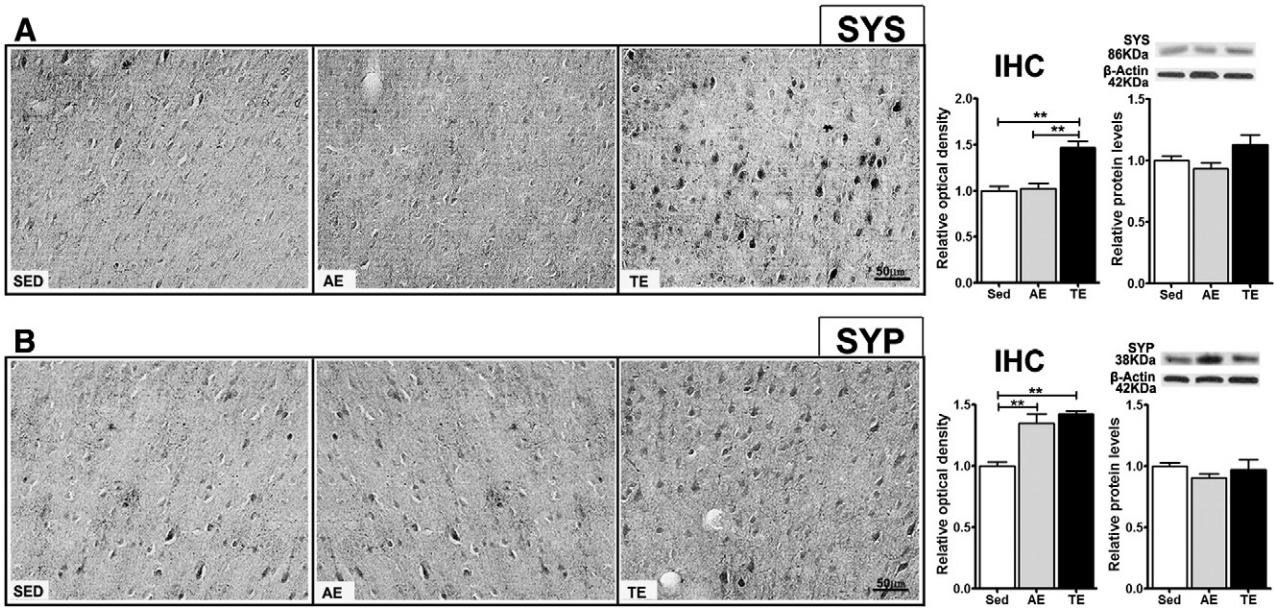

Fig. 3 – Effects of AE and TE on SYS (A) and SYP (B) in the rat motor cortex. Digital images of coronal sections of the motor cortex stained for SYS and SYP. Mean ratio of SYS and SYP integrated density data in the motor cortex (M1 and M2) in relation to the background optical density (N = 6–8 per group). Mean ratio of SYS/β-actin and SYP/β-actin densitometry (N = 7–14 per group). Density data relative to the sedentary group (normalized) and typical immunoblots in each condition. AE: acrobatic exercise; TE: treadmill exercise; Sed: sedentary; SYS: synapsin I; SYP: synaptophysin; IHC: immunohistochemistry (p < 0.03).**

The staining for SYS and SYP in this region exhibited a diffuse punctate pattern of staining throughout the striatum. The intensity of staining for SYS was significantly higher in the dorsomedial striatum in both exercised groups ($p<0.03$). As for the staining of SYP, the intensity was significantly higher in the dorsomedial striatum of the AE group ($p<0.05$) and in the dorsolateral striatum of the TE group ($p<0.03$). The protein analysis of these synaptic proteins revealed increases of SYP after AE ($p<0.05$), whereas SYS remained unchanged. (Fig. 5).

2.3.3. *Cerebellum*

In the cerebellum, we observed a cytoplasmic pattern of staining of the Purkinje cells for both MAP2 and NFs. On the granular and molecular layers we observed staining along the axons and dendrites. This staining of neuropil and perikarya was observed throughout the cerebellum. The MAP2-immunoreactive dendrites were in the innermost region of the molecular layer, proximal to the Purkinje cell dendrites, and extend diffusely throughout the molecular layer, where we observed a higher intensity of staining in the TE group ($p<0.03$). The staining for NFs was also diffusely distributed in the granular layer, where we observed a higher intensity of staining in the TE ($p<0.05$), and in the molecular layer, where we observed a lower intensity of staining in the TE group ($p<0.05$). Protein analysis of the cerebellum revealed an increase of MAP2 levels after TE ($p<0.001$), whereas NF68 levels remained unchanged and AE did not produce changes of either protein in this region (Fig. 6).

As for the synaptic proteins studied, we observed a punctate pattern of staining for both SYS and SYP among the Purkinje cells, and a diffusely distributed staining along the molecular layer. Little staining was observed in the granular layer. We were able to observe higher intensity of staining for SYS in the granular layer of the TE group ($p<0.03$) and in the molecular layer of both AE and TE groups ($p<0.03$). Protein analysis, however, did not show any changes for either synaptic protein in the cerebellum (Fig. 7).

3. Discussion

Our results show that, regardless of the modality, physical exercise was able to induce improvement of motor skills and changes of protein expression in the motor brain areas studied here. However, these changes were distinct for treadmill and acrobatic exercise, varying according to the brain region and protein studied.

3.1. *Behavioral analysis*

Both AE and TE groups increased the time spent on the Rotarod, starting after 2 weeks of exercise, whereas the sedentary group did not increase its scores. This suggests improvement of coordination and balance of these rats in response to AE and TE. This improvement persisted until the end of the exercise protocols. Since acrobatic exercises demand more motor skills, such as balance and coordination, we expected the AE group to spend more time on the Rotarod than the TE group. However, this did not happen, as the TE group presented the same scores as the AE group. We hypothesize that, since the treadmill is a rhythmic activity similar to the cylinders of the Rotarod, and the speed increments of the Rotarod happen only every 30 s, this might not have caused enough instability. Therefore, the Rotarod evaluation might not have been sensitive enough to

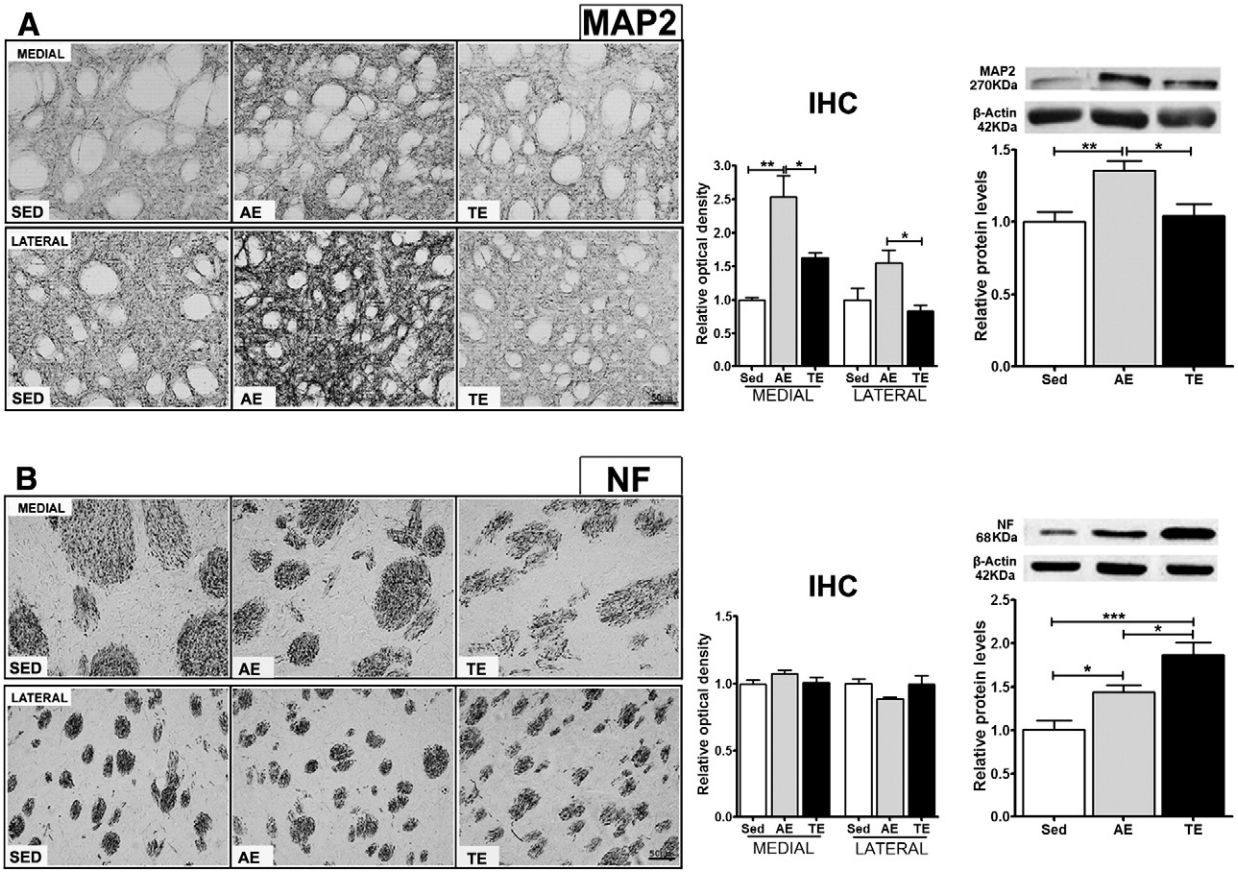

Fig. 4 – Effects of AE and TE on MAP2 (A) and NFs (B) in the rat striatum. Digital images of coronal sections of the striatum stained for MAP2 and NF68. Mean ratio of MAP2 and NF68 integrated density data in the dorsomedial and dorsolateral striatum in relation to the background optical density (N = 6–8 per group). Mean ratio of MAP2/β-actin and NF68/β-actin densitometry (N = 7–14 per group). Density data relative to the sedentary group (normalized) and typical immunoblots in each condition. AE: acrobatic exercise; TE: treadmill exercise; Sed: sedentary; MAP2: microtubule-associated protein 2; NF: neurofilaments; NF68: 68 kDa neurofilament; IHC: immunohistochemistry (*p < 0.05; **p < 0.03; ***p < 0.001).

differentiate the balance and coordination improvements caused by the treadmill and acrobatic exercise.

One of the characteristics that define learning of a motor ability is the improvement of performance to complete the learned task/behavior (Derksen et al., 2007). We observed evidence of further motor learning of our AE rats, as they were able to complete the acrobatic circuit in less time and with less hesitation 2 weeks after the beginning of the protocol. The reduction of the time necessary to complete an acrobatic circuit has also been observed by other groups using different training protocols. Kleim et al. (1998b) trained young adult rats for 30 consecutive days, during which the rats repeated the circuit task 5 times each day and by the 10th day of training they were able to observe a reduction of the time necessary to complete this task. Klintsova et al. (2004), however, trained their rats for 1, 3, 5 or 7 consecutive days, during which the rats repeated the circuit task 3 times each day, and observed that the time necessary to complete the task progressively decreased after the 5th day of training. Furthermore, these previous studies have shown that the reduction of the time necessary to complete an acrobatic circuit is due to the progressive reduction of the number of mistakes along the circuit (misplacement of the paws, deficient motor control) (Kleim et al., 1998b; Klintsova et al., 1998).

3.2. *Changes in protein expression*

The present study also showed that the different types of exercise training induced distinct changes of MAP2, NFs, SYS and SYP in the various brain regions studied. In addition, we could also observe that the structural proteins underwent more general changes in all brain regions studied, as we could find changes in protein levels by Western blotting, whereas the changes of synaptic proteins were detected more frequently by immunohistochemistry, as these were only focal changes. It is noteworthy, that these two methods offered complementary data facilitating a global comprehension.

3.2.1. *Motor cortex*

Our results for the cortex showed increased MAP2 and SYP after AE and decreased levels of NF160 and NF200, and increased SYS and SYP after TE.

Increased MAP2 has also been observed by other groups in regions such as the hippocampus after short-term treadmill

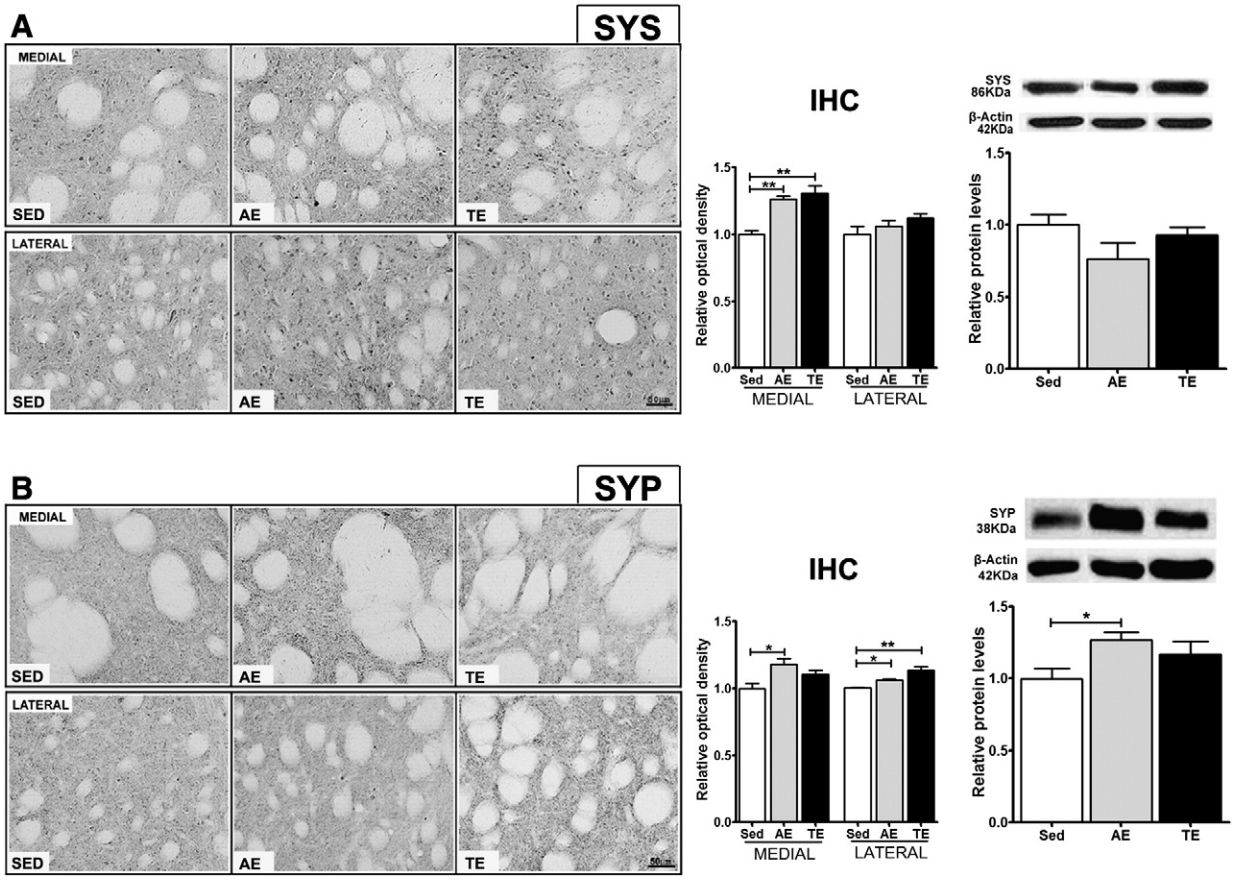

Fig. 5 – Effects of AE and TE on SYS (A) and SYP (B) in the rat striatum. Digital images of coronal sections of the striatum stained for SYS and SYP. Mean ratio of SYS and SYP integrated density data in the dorsomedial and dorsolateral striatum in relation to the background optical density (N = 6–8 per group). Mean ratio of SYS/β-actin and SYP/β-actin densitometry (N = 7–14 per group). Density data relative to the sedentary group (normalized) and typical immunoblots in each condition. AE: acrobatic exercise; TE: treadmill exercise; Sed: sedentary; SYS: synapsin I; SYP: synaptophysin; IHC: immunohistochemistry (*p < 0.05; **p < 0.03).

exercise (Ferreira et al., 2011) and the motor cortex after different types of acrobatic training (Derksen et al., 2007). In the latter study, the authors evaluated the expression of MAP2 in the motor cortex of adult rats submitted to 4 different acrobatic circuits of increasing complexity for 1, 3, 5 and 7 days. They observed that MAP2 protein levels increased, although there was no correlation between the protein levels and the complexity of the circuit. Studies of new motor skill learning revealed a significant increase in the mean area of movement representations (Kleim et al., 1998a) and increased synaptic density within the rodent motor cortex (Jones et al., 1999). Considering that MAP2 is related to dendritic growth and remodeling (Sanchez et al., 2000), the increase of MAP2 and SYP in the motor cortex after acrobatic training can be involved in dendritic arborization (Tucker et al., 1989) and synaptogenesis (Brock and O'Callaghan, 1987), which are consistent with an expansion and higher activation of motor cortex in association with motor skill learning.

There are still scarce data on changes of NFs in response to exercise (Ding et al., 2006; Ferreira et al., 2010, 2011). We did, however, observe decreased protein levels of NF160 and NF200 after TE in the motor cortex. Ferreira et al. (2010) observed decreased levels of NF68 e NF160 after 3 days and increased levels of NF68 after 15 days of treadmill exercise in the motor cortex and brainstem (specifically the reticular formation). Since treadmill exercise should be automated relatively fast and should stimulate other brain regions (Holschneider and Maarek, 2008), this is in accordance with decreased NF expression that support a possible attenuation of the motor cortex activation occurring at a later time due to habituation. On the other hand, increased SYS and SYP in the motor cortex after TE was observed only by immunohistochemistry, due to its high spatial resolution. Both SYS and SYP are associated to neurotransmitter release and are responsible for the formation and anchoring of synaptic vesicles, as well as contributing to a fast and efficient neurotransmission (Vaynman et al., 2006).

In summary, increased MAP2 and SYP in the motor cortex (M1 and M2) after AE and decreased NFs and increased SYS and SYP after TE could be interpreted as distinct cortical reorganization processes influenced by different types of motor activity. AE induced an increased plasticity, possibly involving increased dendritic arborization (Sanchez et al., 2000) and synaptogenesis (Brock and O'Callaghan, 1987). This finding may be related to the involvement of the motor cortex in the planning and execution of complex movements during AE. On the other

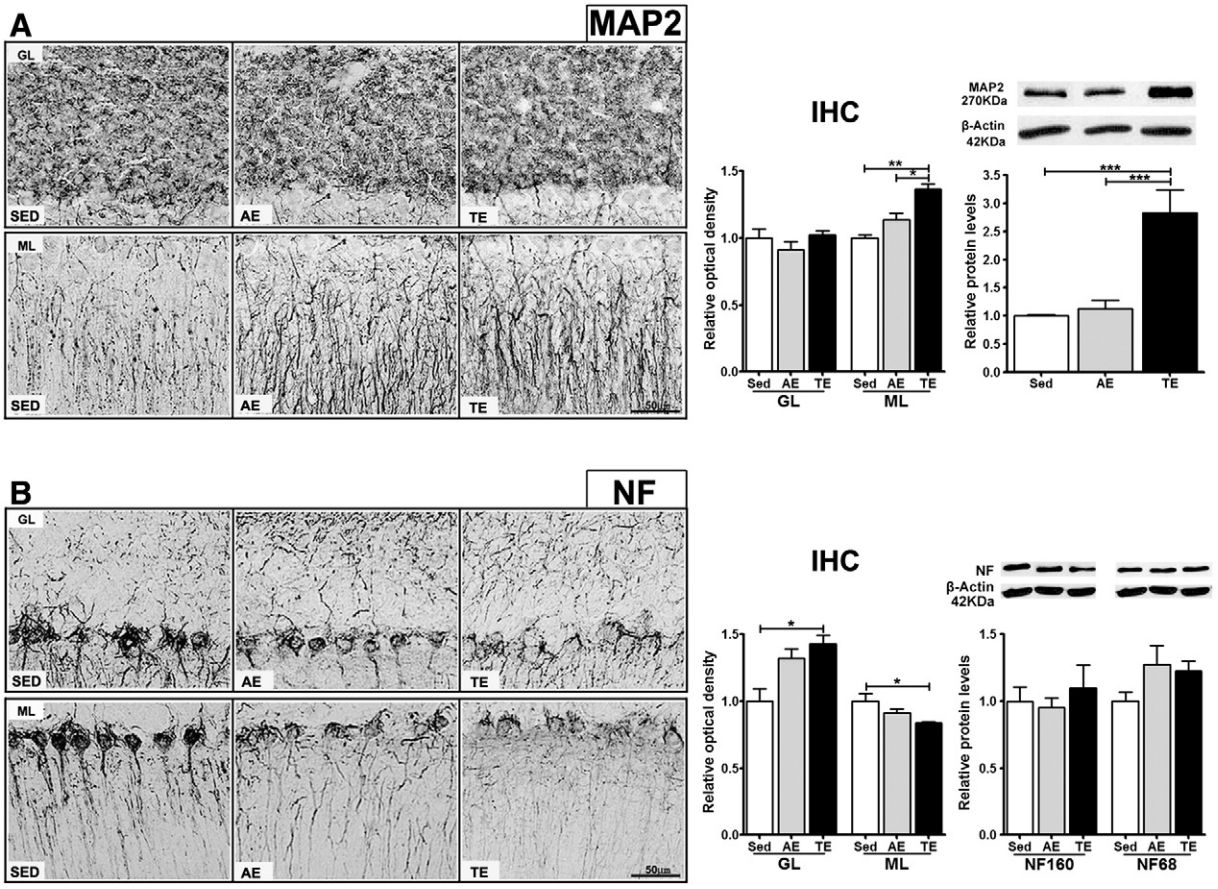

Fig. 6 – Effects of AE and TE on MAP2 (A) and NFs (B) in the rat cerebellum. Digital images of coronal sections of the cerebellar cortex stained for MAP2 and NFs. Mean ratio of MAP2 and NFs integrated density data in the granular and molecular layers in relation to the background optical density (N = 6–8 per group). Mean ratio of MAP2/β-actin, NF68/β-actin and NF160/β-actin densitometry (N = 7–14 per group). Density data relative to the sedentary group (normalized) and typical immunoblots in each condition. AE: acrobatic exercise; TE: treadmill exercise; Sed: sedentary; MAP2: microtubule-associated protein 2; NF: neurofilaments; NF68: 68 kDa neurofilament; NF160: 160 kDa neurofilament; IHC: immunohistochemistry (*p < 0.05; **p < 0.03; ***p < 0.001).

hand, TE is a rhythmic and automatic activity and may need mainly the motor cortex activation for the execution of movements, which thus exhibit axonal remodeling (Perrot et al., 2008) and increased synaptic efficiency (Fornasiero et al., 2010). These processes may involve decreased NF and increased SYS and SYP, respectively.

3.2.2. *Striatum*

Moderate treadmill exercise, as well as protocols designed to promote learning of new motor skills, are widely used to demonstrate plastic changes in the striatum (Ferreira et al., 2010; Lambert et al., 2005; Meeusen et al., 1997) and other regions of the rodent brain (Kleim et al., 2002; Klintsova et al., 1998; Real et al., 2010). The present study showed that both TE and AE induced more robust changes of structural proteins then of synaptic proteins in the striatum. AE increased MAP2 and NF68, whereas TE increased NF68 in the striatum. It is noteworthy that MAP2 and NF68 have important functions in the brain, as NF68 ensures structural support to axons and MAP2 participates in the growth and branching of dendrites, establishing and maintaining synaptogenesis (Sanchez et al., 2000). Some studies suggested that the striatum is involved in initial phases of motor task learning and in later phases of movement automation (Miyachi et al., 2002; Yin, 2010). Thus, the differential pattern of the expression between AE and TE may support these distinct striatum roles in the acquisition of complex motor skill and consolidation or automation of the rhythmic motor activity, respectively.

The only change of synaptic protein observed by protein analysis was an increase of SYP after AE in the striatum. We did, however, observe local immunostaining increases of SYS and SYP in both exercised groups. In the striatum, we could observe increased SYP expression in the dorsomedial striatum (involved in complex motor control and learning of motor tasks) after AE and in the dorsolateral striatum (involved in consolidation and motor automation) (Miyachi et al., 2002; Yin, 2010) after TE, whereas SYS increased after AE and TE only in the dorsomedial striatum. Both SYS and SYP are associated to neurotransmitter release and are responsible for the formation and anchoring of synaptic vesicles, as well as contributing to a fast and efficient neurotransmission (Vaynman et al., 2006). Other studies have observed changes of SYS levels after

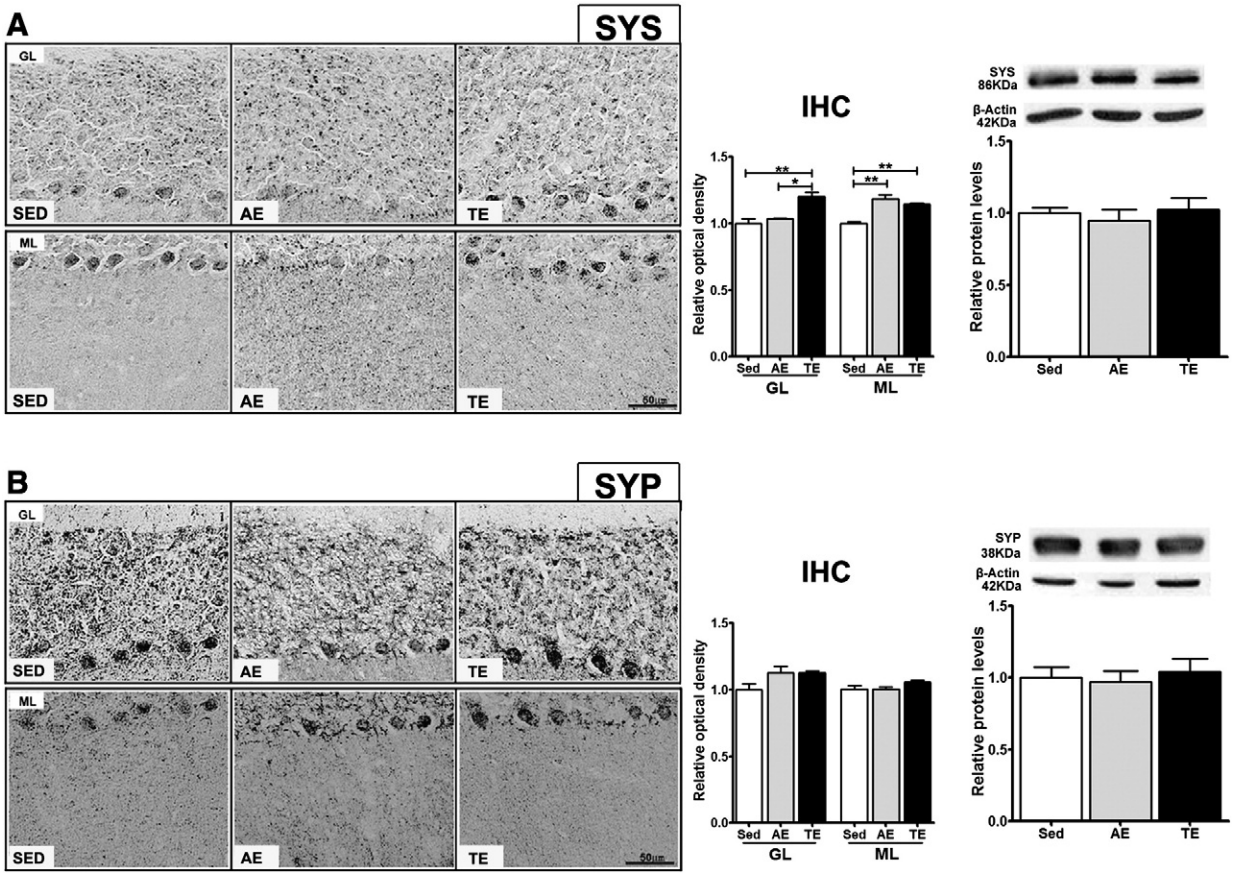

Fig. 7 – Effects of AE and TE on SYS (A) and SYP (B) in the rat cerebellum. Digital images of coronal sections of the cerebellar cortex stained for SYS and SYP. Mean ratio of SYS and SYP integrated density data in the granular and molecular layers in relation to the background optical density (N = 6–8 per group). Mean ratio of SYS/β-actin and SYP/β-actin densitometry (N = 7–14 per group). Density data relative to the sedentary group (normalized) and typical immunoblots in each condition. AE: acrobatic exercise; TE: treadmill exercise; Sed: sedentary; SYS: synapsin I; SYP: synaptophysin; IHC: immunohistochemistry (*p < 0.05; **p < 0.03).

different exercise protocols. After short-term treadmill exercise, increased SYS levels are observed in the striatum (Ferreira et al., 2010). SYS has also been shown to increase after 3 days of voluntary exercise in the hippocampus (Vaynman et al., 2006). Taken together, these data suggest that SYS may be involved in the initial phases of exercise.

Finally, the differential pattern of structural and synaptic protein expression induced by AE and TE in the striatum corroborates with previous studies that have shown distinct functional roles of the dorsomedial (or associative) striatum and dorsolateral (or sensorimotor) striatum, based on their major inputs (Yin, 2010).

3.2.3. *Cerebellum*

Our results showed that the cerebellum was influenced almost exclusively by TE, resulting in increased MAP2 protein levels and higher staining for NFs and SYS, with the only effect of AE being an increased pattern of staining of SYS restricted to the molecular layer. Black et al. (1990) have shown that TE rats had a greater density of blood vessels in the molecular layer than did either AC or inactive animals, suggesting that increased synaptic activity elicited compensatory angiogenesis. Our results showed an increase of MAP2 levels and increased staining for SYS induced by TE, which can both be substrates for an intense synaptic activity observed in the cerebellar molecular layer (Black et al., 1990). In fact, increased arborization (Tucker et al., 1989), may result in increased synaptic contacts and increased neurotransmitter release (Fornasiero et al., 2010). However, neither of the present exercise protocols induced changes of NF levels in the cerebellum, even though we could observe an increased staining pattern in the granular layer and decreased staining pattern in the molecular layer after TE. This might suggest that the NF68 response is more important after shorter periods of exercise (Ferreira et al., 2010), and that after 30 days this protein has already returned to control levels.

Electron microscopy studies have also shown that animals trained for 30 consecutive days on a complex motor learning task had increased number of synapses per Purkinje cell within the cerebellar cortex (Black et al., 1990; Kleim et al., 1997). These studies showed an increased pattern of staining for SYS in the molecular layer after AE, which can be interpreted as increased synaptic efficiency in this region in relation to its participation in balance and motor coordination. The fact that SYS has an important role in neurotransmitter release supports this assumption (Fornasiero et al., 2010).

3.3. *Motor training and functional circuits*

The present study showed that the different types of exercise training induced distinct changes on the motor circuitries analyzed. AE increased MAP2 and SYP in the motor cortex and increased MAP2, NF68, SYS and SYP in the striatum, suggesting that this type of exercise targeted the basal ganglia-thalamic-cortical circuit in detriment of the cerebellum. On the other hand, the cerebellum-thalamic-cortical circuit was influenced almost exclusively by TE, resulting in increases of MAP2, NFs and SYS in the cerebellum, with the only effect of AE being an increased pattern of staining for SYS, restricted to the molecular layer. A distinction has been made between tasks that require a high amount of attentional guidance (i.e. skilled training) and those that do not (overlearned, automatic, or endurance based), and each has been linked with different patterns of functional brain activation (Adkins et al., 2006). Lewis et al. (2007) stated that for internally guided movements, such as acrobatic exercises, there is a predominant activation of basal ganglia-thalamic-cortical circuits. As learning progresses, movements are refined and become automatic (therefore with an externally guided control) and there is a predominant activation of cerebellar-thalamic-cortical circuits, which would be the case of treadmill exercise. These circuits work together in balance and are more or less activated depending on the task (Lewis et al., 2007). Other authors support this theory, such as Holschneider et al. (2007), who showed that treadmill exercise for 6 weeks increases cerebral blood flow in areas of the cerebellar-thalamic-cortical circuit. Furthermore, the increase of SYS in the molecular layer of the cerebellar cortex after AE possibly suggests the start of a shift to a predominantly activated cerebellar-thalamic-cortical circuit, since the acrobatic circuit may become automatic after more than 30 days of training.

4. Conclusion

Our results show that both TE and AE induce improvements of motor behavior and plasticity. Acrobatic exercise induced increases of structural and synaptic proteins in the motor cortex and striatum, supporting the involvement of these regions in the process of learning new skills. Treadmill exercise, on the other hand, produced a more diffuse effect, increasing structural and synaptic proteins in the motor cortex, striatum and cerebellum. These results confirm that the type of exercise is a determining factor of which brain regions are more affected and suggest that different proteins participate in the underlying plasticity, even when the behavioral outcome is the same. The understanding of the relationship between these different types of exercise, mechanisms of neural plasticity, and changes in motor behavior may facilitate the development of more effective rehabilitation interventions.

5. Experimental procedures

5.1. *Animals*

Male 2 month-old, young adult *Wistar* rats weighing ca. 250 g (obtained from the Animal Facility of the Institute of Biomedical Sciences of the University of São Paulo) were housed in groups in standard polyethylene cages with food and water ad libitum, room temperature of 23 °C and a 12/12 h light–dark inverted cycle (Holmes et al., 2004). Animals were randomly divided into three groups: treadmill exercise (TE) (n=20), acrobatic exercise (AE) (n=20) and sedentary (Sed) (n=15). All protocols were approved by the Ethics Committee for Animal Research of the University of São Paulo and experimental procedures were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and the animal care guidelines of the National Institutes of Health (NIH/USA).

5.2. *Evaluation of balance and coordination*

Before the rats went through their designated exercise modality, an initial evaluation of motor skills was conducted. This evaluation was then repeated after 2 weeks and then again after 4 weeks of the exercise period for all three groups. We chose to use a Rotarod, as this instrument allows us to evaluate balance and coordination (Brooks and Dunnett, 2009). The Rotarod was initially designed to measure neurologic deficits in rodents and represents the most commonly used instrument to test motor function in rats (Brooks and Dunnett, 2009). The rats are placed on top of the 4 individual cylinders (7 cm-diameter) which rotate at a progressively increasing speed (increments every 30 s). Once the rat falls from the cylinder, it lands on a pressure-sensitive platform which records how long the rat stayed on the cylinder.

5.3. *Acrobatic exercise protocol*

An acrobatic circuit was built based on what was used by Black et al. (1990), which constituted of the following obstacles placed at a height of 1.5 m from the ground: see-saw, balance beams, rope ladder, thin dowels and rope bridge. All animals went through a two-day adaptation period to the acrobatic task during which they passed through the entire circuit twice each day. The acrobatic task was performed 3 times a week for a period of 4 weeks, during which the rats were allowed 5 trials each day. When necessary, rats were gentle guided manually to continue to next obstacle. The time spent to complete the task on each trial was recorded (Black et al., 1990; Kleim et al., 1996). Platforms and bridges were cleaned with 70% ethanol after each rat completed its session.

5.4. *Treadmill exercise protocol*

All animals went through a two-day adaptation period to a treadmill (KT 3000 — IMBRAMED, Brazil, adapted for rats) during which they were allowed to explore the equipment and the treadmill was turned on for only 15 min at low speeds (0.3 to 0.5 km/h). This procedure has the purpose of excluding animals which are intolerant to the treadmill and refuse to run, providing a homogenous group of rats for the TE group. These rats exercised for 40 min a day, 3 times a week at 10 m/min (0.6 km/h) in the middle of the active cycle (between 11 am and 1 pm), whereas the sedentary group remained in the cages near the treadmill. The inverted cycle and that period of training were used to avoid the development of internal desynchronization,

similar to the effect observed in night-shift workers, which was previously detected in rats that exercised during their light cycle (Salgado-Delgado et al., 2008).

5.5. *Immunohistochemistry*

5.5.1. *Tissue processing*

One hour after the exercise period, the animals (4 animals per group) were deeply anesthetized (ketamine, 20 mg/100 g of body weight; xylazine, 2 mg/100 g, i.m.) and perfused transcardially with 300 mL of 0.1 M phosphate buffered saline (PBS) followed by 300 mL of 2% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4. The brains were then removed and post-fixed for 4 h in the same fixative at 4 °C and cryoprotected with a 30% sucrose solution (in PB) for 48 h at 4 °C. Coronal sections (30 μm) were cut on dry ice using a sliding microtome (Leica SM 2000R — Nussloch, Germany). Sections were stored in PB at 4 °C until use.

5.5.2. *Immunostaining*

Free-floating sections were stained with a series of antibodies, namely rabbit polyclonal anti-SYS (1:1000) (Chemicon, Temecula, USA), rabbit polyclonal anti-SYP (1:250) (DakoCytomation, Glostrup, Denmark), mouse monoclonal anti-NFs (PAN, recognizing 68 kDa, 160 kDa and 200 kDa neurofilaments) (1:2000) (Zymed Laboratories, San Francisco, CA, USA) and mouse monoclonal anti-MAP2 (1:1000) (Chemicon, Temecula, USA). All antibodies are routinely used by several laboratories. The secondary antibodies were biotinylated goat anti-rabbit antisera for SYS, donkey anti-rabbit antisera for SYP, donkey anti-mouse antisera for MAP2 (all from Jackson Immuno Research Lab., West Grove, Pennsylvania, USA) and a goat anti-mouse antiserum for NFs (Vector, Burlingame, CA, USA). The primary antibodies were diluted in PB with 0.3% Triton X-100 and 5% normal goat serum (for anti-SYS and anti-NFs) or normal donkey serum (for anti-SYP and anti-MAP2) and the sections were incubated overnight (14–20 h) at room temperature (ca. 24 °C). After washing the sections with PB (3×10 min), they were incubated with the corresponding secondary antibodies, which were all diluted 1:200 in PB with 0.3% Triton X-100 for 2 h at room temperature. Following additional washes (3×10 min), the sections were incubated with the avidin–biotin–peroxidase complex (ABC Elite kit, Vector Labs., Burlingame, CA, USA) for 2 h at room temperature. Labeling was developed with 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.03% (final concentration) hydrogen peroxide in PB. To confirm the specificity of the antibodies, a separate set of sections from each group was incubated only with the secondary antibodies, a condition in which no staining was present. After the staining procedure, the sections were mounted on glass slides and the staining was intensified with 0.05% osmium tetroxide in water. They were then dehydrated and coverslipped using Permount (Fisher, Pittsburg, PA, USA). The region of interest was identified based on a stereotaxic atlas (Paxinos and Watson, 2005) and the corresponding images captured using a Nikon DMX1200 digital camera and quantified by using integrated optical density, normalized to the background optical density, measured in the same way in the same section with the aid of Image J (NIH/USA). For primary (M1) and secondary (M2) motor cortex we analyzed 8 areas of 540.000 μm^2 of the motor cortex for each rat between the planes designated as bregma 2.52–1.56. For the striatum we analyzed 12 areas of 300.000 μm^2 from dorsomedial and dorsolateral areas for each rat between bregma 1.80–0.36, and for the cerebellum we analyzed the cerebellar cortex (paramedian lobule) in 8 areas of 160.000 μm^2 for each rat.

5.6. *Western blotting*

One hour after the exercise period, the animals (12 animals per group) were deeply anesthetized (ketamine, 20 mg/100 g of body weight; xylazine, 2 mg/100 g, i.m.), decapitated and regions of interest were quickly collected, frozen in liquid nitrogen and stored at −70 °C until use. The tissue was then homogenized at 4 °C in extraction buffer (Tris, pH 7.4, 100 mM; EDTA 10 mM; PMSF 2 mM; aprotinin 0.01 mg/ml). The homogenates were centrifuged at 12,000 rpm (15,294 *g*) (Eppendorf Centrifuge 5804R — Westbury, NY, USA) at 4 °C for 20 min, and the protein concentration of the supernatant was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) (Bradford, 1976). The material was stored in a sample buffer (Tris/HCl 125 mM, pH 6.8; 2.5% (p/v) SDS; 2.5% 2-mercaptoethanol, 4 mM EDTA and 0.05% bromofenol blue) (Laemmli, 1970) at −70 °C until starting the assays. Samples containing 100 μg of total proteins in Laemmli buffer were boiled for 5 min and separated by 6.5% and 8% acrylamide SDS gels (Bio-Rad, Hercules, CA, USA) at 25 mA (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membranes (Millipore, Temecula, CA, USA) at 100 V for 80 min using a Trans-Blot cell system (Bio-Rad, Hercules, CA, USA). The membranes were then blocked for 2 h at room temperature with PBS containing 0.05% Tween-20 (TTBS) and 5% non-fat milk, and incubated overnight at 4 °C with the same primary antibodies used for immunohistochemistry at a concentration of 1:1000. The membranes were then incubated for 2 h with anti-rabbit-HRP IgG for SYS and SYP, and anti-mouse-HRP IgG for NFs and MAP2 (Amersham, Little Chalfont, Buckinghamshire, UK) diluted 1:10,000 in TTBS with 1% non-fat milk. The probed proteins were developed by using a chemiluminescent kit (ECL, Amersham Biosciences, NJ, USA). The membrane was then incubated for 30 min at room temperature with stripping buffer and an anti-β-actin antibody (Sigma, St. Louis, MO, USA) was used to quantify β-actin as a loading control. The bound antibodies were visualized using radiographic films which were placed in contact with the membranes, then developed and fixed. The quantification of band intensity was performed with Image J (NIH Image). Subsequently, the normalized data were treated to evaluate protein changes in the experimental structures in relation to the controls.

5.7. *Statistical analysis*

Data are expressed as the mean ± SEM. Statistical analyses were performed using one-way ANOVA with Tukey *post-hoc* test for behavioral, immunohistochemistry and Western blotting data. The significance level used was 5%.

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